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Cell Wall Deficiency in Mycobacteria: Latency and Persistence

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1. Introduction

It is believed that persistence of small populations of *Mycobacterium tuberculosis* in hosts underlies latent tuberculosis. Very little is known about the morphological and physiological nature of tubercle bacilli in latent TB. It is under discussion whether and how tubercle bacilli adapt to latent state and remain alive in face of damaging stressful conditions such as antibiotics and host immune factors. In this respect, cell wall deficiency (existence without rigid walls) in mycobacteria and its occurrence *in vivo* suggests one of the possible pathways by which tubercle bacilli can survive, replicate and persist within the body for a long period, harboring latent tuberculosis with a risk for disease reactivation, in case of reversion to classical TB bacilli upon changes in host immune status. Essentially, cell wall deficiency, or the ability of bacteria to exist as populations of self-replicating forms with defective or entirely missing cell walls (L-forms), is considered an adaptive strategy of bacteria to survive and reproduce under unfavorable circumstances.

This chapter elaborates on some special aspects of the L-form phenomenon and its importance for discovering new fundamental aspects of TB bacillary morphology and physiology, as well as understanding the mechanisms of latent tuberculosis.

2. History

L-forms were first observed by Emmy Klieneberger-Nobel, in 1945, whose typical "fried eggs"-shaped colonies, duplicating Mycoplasma, were isolated from cultures of *Streptobacillus moniliformis.* The wall-less variants of L-forms she named after the institution she worked in – England's Lister Institute.

Fig. 1. Emmy Klieneberger-Nobel – the founder of bacterial L-forms.

The period between 1882 and 1940, after Robert Koch discovered the cause of tuberculosis, was marked by series of papers reporting about the appearance of L-form elements in cultures of mycobacteria, such as filterable forms, branching filaments, syncytial growth, large spheres and "variegated mycelia", all of which characterize mycobacterial growth. Mattman summarized the known data about the ability of *M. tuberculosis* to convert to cell wall deficient forms and suggested a "L-cycle" for mycobacteria (Mattman et al., 1960; Mattman, 1970, 2001).

Fig. 2. Lida Mattman

Despite the long history in tuberculosis research, the nature of cell wall deficiency and its association with persistence in life of mycobacteria still remain obscure. Unfortunately, over the last several decades, investigations on these unusual forms of tubercle bacilli have been ignored and neglected. Information about forming of mycobacterial L-forms *in vitro* (in the laboratory), as well *in vivo* (within the body) is based mainly on studies concerning their morphological appearance. Two periods in L-form research of mycobacteria should be distinguished: before introduction of chemotherapy against tuberculosis, and after. Observations made in the beginning of 20th century on mycobacterial pleomorphism and L-form elements provide evidence for existence of L-forms without contact with antimicrobial drugs (Calmette & Valti, 1926; Much, 1931). In the following decades, examinations regarding modification of morphology and L-form transformation by antimicrobials became the starting point of additional information on mycobacterial properties (Dorozhkova & Volk, 1972; Dorozhkova & Volk, 1973; Kochemasova et al., 1968; Mattman et al. 1960; Wang & Chen, 2001).

3. Basic characteristics of cell wall deficient L-forms

3.1 L-conversion, morphology and ultrastructure

Bacterial L-form conversion, i.e. existence without rigid walls, is universal but difficultly recognized phenomenon in nature (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981). The term „cell wall deficiency" implies alterations in the constitution of bacterial cell wall, resulting from deletion and faulty synthesis of wall components (Mattman, 2001). It is considered that imbalance of cells' ability to degrade and synthesize its classical thick wall results in cell wall deficiency. Since the peptidoglycan is the stress-bearing structure of bacteria, its loss, respectively the loss of rigidity, is a distinctive characteristic of cell wall deficient forms (L-forms). In fact, morphological variability is an indicative and common feature of all L-forms, regardless of what bacterial species they originated from. Although these forms have been observed in patients' specimens for many decades, most are ignored and generally regarded as diagnostically insignificant staining artifacts or debris

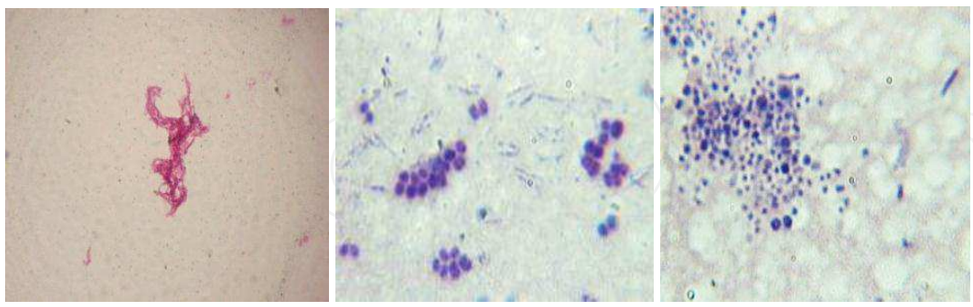
(Domingue, 2010). It is assumed that these pleomorphic forms represent various stages in the life cycle of stressed bacteria.

M. tuberculosis is known to exhibit extreme pleomorphism in certain circumstances. Various morphological forms of mycobacteria were observed by many authors and were described as "mycococcus form" (Csillag, 1964), large "amoeba-like cells" (Imaeda, 1975), giant non-cellular structures or so called "budding yeast-like structures" (Koch, 2003), "elementary bodies and filament structures" (Merkal et al, 1973) "endospores" (Ghosh et al.,2009; Traag et al, 2010) and "ovoid cells"(Shleeva et al., 2011). Mycobacteria are unique among procaryotes with their cell wall structure, containing tightly packed mycolic acids that provide TB bacilli with efficient protection and remarkable capacity to resist to various exogenous stress conditions. The high concentration of lipids in cell wall of mycobacteria is associated with general insusceptibility to chemical/toxic agents and most antibiotics. The mycolic acids and glycolipids in cell wall of mycobacteria also impedes the entry of nutrient substrates, causing the organisms to grow slowly (Draper, 1998). However, mycobacterial cell wall appears to be a dynamic structure that can be remodeled, as the microorganism is either growing, or persisting in different environments (Kremer & Besra, 2005). Under unfavorable conditions, where mycobacteria are exposed to different damaging factors particularly in face of host defense mechanisms, they may produce cell wall deficient forms (L-forms) (Markova et al.2008a; Markova et al. 2008b). A variety of papers reported about production of mycobacterial L-forms experimentally *in vitro,* using different inducing factors. Wide range of substances (cell wall inhibitors) as antibiotics, lytic enzymes and some amino acids affecting cell wall and especially biosynthesis of peptidoglycan have been used as L-inducing factors (Beran et al., 2006; Hammes et al., 1973; Hines and Styer, 2003; Naser et al., 1993; Udou et al., 1983). Indeed, it is important to understand how mycobacteria regulate the cell wall composition in response to changing environment. In some wall deficient cells pieces of cell wall are synthesized and dutifully pulled through the pores of cell membrane but somehow lack structural detail that would permit them to link together. Mitchel & Moyle have added another interesting aspect to consider, which may explain why a cell is unable to resynthesize its cell wall, once losing it. They postulate that perhaps the building blocks are sufficiently soluble to diffuse spontaneously into the culture medium than remain together against the wall where their union is facilitated (Mitchel & Moyle, 1956).

The ability of strains from *M. tuberculosis* complex to produce L-phase variants after nutrient starvation stress was demonstrated in our experiments (n. d.). Morphological transformations of tubercle bacilli from acid fast to polymorphic non-acid-fast and coccoid forms of varying size were observed (Fig. 3). In contrast to classical tubercle bacilli, which typically appear as straight or slightly curved red stained rods in Ziehl-Neelsen stained smears, mycobacterial L-forms showed marked polymorphism and variability in staining reaction. L-form variants of mycobacteria lost acid fastness completely and resembled the morphology of various other bacteria (Fig. 3 b, c).

It is known that acid fastness is dependent on the integrity of the tubercle bacilli. Sometimes, persistent *M. tuberculosis* bacteria bearing cell wall alterations may remain undetected by the classic Ziehl-Neelsen staining (Seiler et al, 2003). Appearance of polymorphic non-acid fast forms and coccoids in cultures of mycobacteria has been observed by other authors

(Chandrasekhar & Ratnam , 1992; Csillag, 1964; Juhasz, 1962; Miller, 1932; Xalabarder, 1958).



a b c

Fig. 3. Ziehl-Neelsen stained smears: (a) control TB bacilli; (b, c) non-acid fast polymorphic cells of *M. tuberculosis* L-forms (n.d.)

Morphological forms of different sizes and shapes (short coccobacilli and long rods, oval or round coccoid cells, large spherical bodies and giant filaments) in mycobacterial L-form cultures obtained after starvation stress, were observed by us with scanning electron microscopy (Fig.4, n. d.). Very small granular elements placed on membrane filters with pore size diameter of 0.22µm, evidencing their ability to pass through bacterial filters i.e. filterable L-form cells, were detected (Fig. 4 f). The filterable forms are considered as minimal reproductive cells, which can be formed from large L-bodies in all possible ways. It is believed that such filterable bodies contain a bacterial genome and minimal metabolic

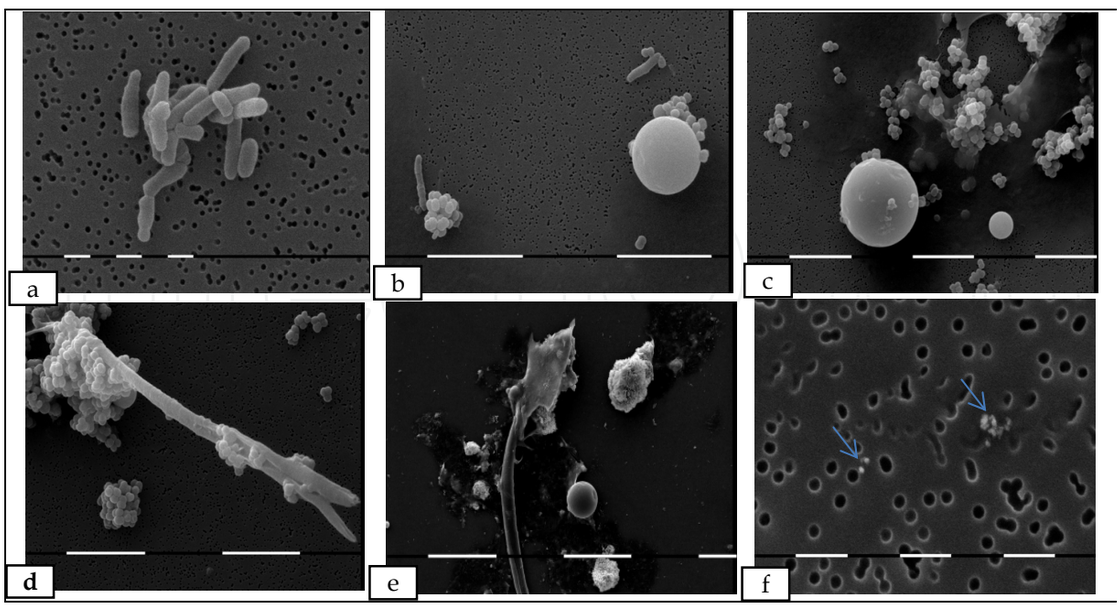


Fig. 4. Scanning electron microscopy of classical tubercle bacilli (a) and mycobacterial L-forms obtained after stress treatments *in vitro* of *M.tuberculosis* (b), *M.bovis* (c, d) and *M.bovis* BCG (e, f), (n.d.).

capability sufficient to initiate reproduction (Domingue, 2010; Klieneberger-Nobel, 1951; Prozorovski et al., 1981).

Findings from transmission electron microscopy yielded additional valuable information about the ultrastructure morphology of mycobacterial L-forms. Examinations of *M. tuberculosis* L-forms obtained *in vitro* after starvation stress (n.d.) or during experimental infection in rats (Markova et al., 2008a) revealed typical fine structure of L-form population. L-phase growth consisted of cells of variable shape and size, completely devoid of bacterial cell wall and bound only by a single unit membrane (Fig. 5). Large and elementary bodies of different electron microscopy density, as well as very small granules and vesicular forms were observed (Fig. 5 b, c, d). Some vesicular forms either appeared empty or contained electron-dense granules (Fig. 5 c, f). Of considerable interest was the observation of large bodies of so called "mother" cells, filled with numerous small spherical L-elements (Fig. 5 f). Such "mother" cells are often internally vesiculated and may produce also small, empty bodies, or membrane bound vesicles. Fragmentation of the cytoplasmic mass in numerous granular forms was the mode of L-form reproduction that was noted. Cytoplasmic condensation at the periphery of the large bodies ending in formation of protrusions and buds was often seen. Budding, another mode of L-form replication, was observed as well. It should be noted that nucleoid and ribosomal areas within L-bodies were of variable electron densities and intracellular location .The nucleoids were variable, being sometimes compact and sometimes scattered throughout the cytoplasm. Enucleated L-bodies were also seen. Ribosomes were either packed together or diffusely scattered, usually at the periphery of the cells. Electron-dense L-bodies of different size and giant filamentous forms were found in clinical isolates of *M. tuberculosis* (Fig.5 g, h; Michailova *et al*, 2005).

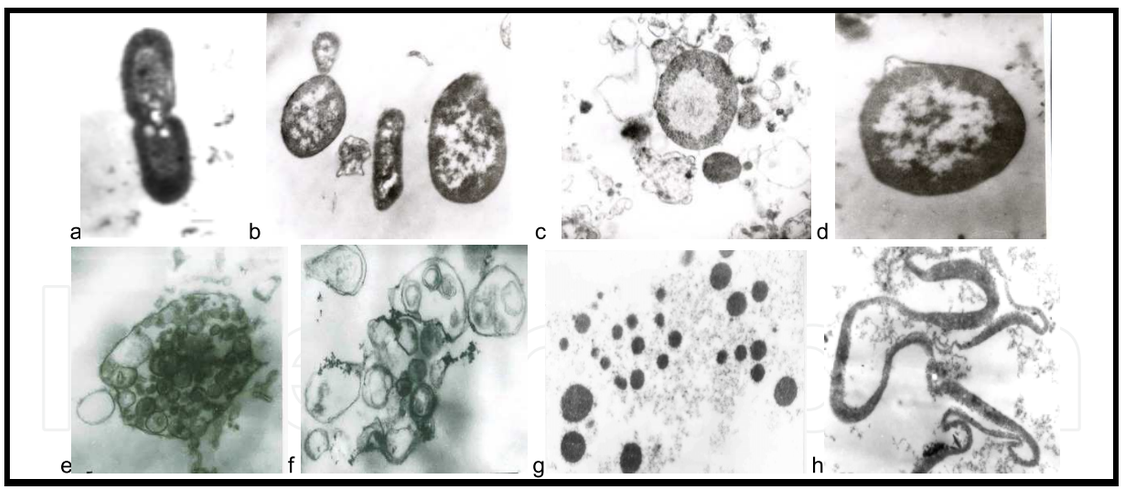


Fig. 5. Transmission electron microscopy of classical tubercle bacilli (**a**), and L-forms of *M. tuberculosis* obtained *in vitro* after starvation stress (**b ,c ,d**; n. d.), isolated from rats, experimentally infected with *M. tuberculosis* (**e, f**; Markova et al.,2008a) and observed in clinical strains, isolated from patients (**g, h;** Michailova et al., 2005).

3.2 Modes of reproduction and morphogenesis of L-cycle

The normal existence of bacteria appears to be a dynamic state of morphological and physiological changes, and the reproducibility in response to established conditions for

growth is considered as a "life style". Under certain circumstances, bacteria can enter unbalanced growth and undergo complex life cycles, involving different morphological transformations, known as the bacterial L-cycle. Conversion to L-forms is assumed to be a general property of bacteria and as adaptive reaction to unfavourable environmental factors, which interfere with the normal reproduction, as well as permit the growth of cell wall deficient variants (Dienes & Weinberger, 1951). Loss of rigidity due to the lack of murein layer in L-forms, results in uncoordinated propagation and appearance of highly pleomorphic forms. In contrast to classical bacteria, L-forms can reproduce by great variety of unusual modes, such as irregular binary fission, budding, protrusion-extrusion of elementary bodies and granules from large bodies, multiple division with intracellular fragmentation of cytoplasm or combination of all types (Prozorovski et al., 1981). Variations in the development of morphological units of different sizes and shapes, typical for L-forms, appear in accordance with the changing environmental conditions (Markova et al, 2010). The newly reorganized L-form population continues to exist and replicate by unusual modes, displaying various cells and elements such as elementary and large spherical bodies, granular and filterable forms, vesicular and empty bodies, giant filaments and others. Those giant filaments and large bodies may be serving a two-fold purpose, playing a role in L-form reproduction, as well as protecting them from unfavourable environment.

Our observation of giant L-bodies ("mother" cell) within mycobacterial L-form population releasing, through protrusion or budding, numerous previously generated granules, is also perhaps noteworthy of mentioning. Such granular elements, often released from the terminal sides of filaments (Fig 4 d, e; n. d.), were found to develop into bigger coccoid or large L-bodies, although transformation of granules into rod shaped forms was also noticed. The segmentation of L-bodies and breaking up into small elements, which germinate again, as well as the processes of regeneration initiated by the fusion of certain elements (Klieneberger-Nobel, 1951), challenge the conventional vision about bacterial replication. Although the modes of L-form replication were less effective, it should be noted that, at a point of their development and adaptation, L-forms started multiplying with remarkable rapidity, by releasing numerous small granules from collapsing giant L-structures. The small forms grew into large bodies which subsequently either increased in diameter, or disintegrated into even smaller L-form bodies. The observed by us different arrangements of *M. tuberculosis* L-forms coccoid cells of varied size (singly, in pairs or in irregular clusters) suggest either capability of L-forms to divide in different planes by binary fission or the possibility that they arose *en masse* from huge L-form bodies. In our opinion, L-life style is best understood by taking into consideration the unusual modes of replication, exhibited by L-forms. L-forms behave like an entire population, within which the role of individual organisms and organelles is difficult to determine (Markova et al., 2010). Of all structures in the L-cycle, syncytium, designed as "symplasm" and consisting of numerous nuclei embedded in a cytoplasm within one L-body (Mattman, 2001), is the most incredible. As noted by Mattman, fifty mycobacteria can be made within one sac (L-syncytium). Syncytia were observed to be formed from coalescing aggregates of bacteria, when the cell walls disintegrate and the cytoplasm starts to coalesce. The granules emerging from the symplasm grow into young cells, which reproduce further by fission or by other modes. According to Norris, syncytium-like structures may create a favorable environment for development of a complex prebiotic ecology, in which rearranged hyperstructures give rise to even more complex life forms (Norris, 2011).

It is assumed that cell wall deficient bacterial forms survive storage and unfavorable conditions much longer than classical bacteria (Mattman, 2001). Domingue suggests the role of small electron-dense bodies (filterable granules) as notoriously resistant forms of pathogenic bacteria (Domingue, 1997). Xalabander noted that L-forms of mycobacteria were remarkably different from L-forms of other species in their resistance to physical and chemical agents. Similar to prions, mycobacterial L-forms escape destruction by body's immune system, and are seemingly imperishable. Xalabander also noted that these L-forms contain both RNA and DNA proteins, but do not stain well by ordinary mycobacteria dyes (Xalabander, 1958; 1963). On other hand, it is supposed that the smallest and most resistant to environmental stresses filterable L-granules, containing DNA may exert nuclear functions (Klieneberger-Nobel, 1951). Moreover, chromosomal DNA, especially within L-symplasm, should be regarded as a substantial mass of the nucleoid body, which can dynamically interact with other components (Allan et al, 2009). This problematic question is still under discussion and yet, no matter how small and at first glance, enucleated, some of these L-forms will revert back to virulent mycobacteria.

Shleeva et al. (2010) believe that dormancy in mycobacteria is related to the formation of different cell forms with various characteristics (less differentiated cyst-like forms, weakly differentiated resting cells and highly differentiated spore-like forms) within a population. According to the same authors, passing into a dormant state is associated with drastically decreased metabolic activity of cells, enhanced resistance to harmful factors, and absence of cell division. The resting cells retain their viability but lose capacity for germination and growth, becoming "nonculturable". It is a generally accepted postulate that TB bacilli are in a true dormant state, undergoing no replication. Dormant cells switch on the mechanisms of division arrest and may persist, due to survival of a small number of bacteria (Kaprelyants et al., 1993; Postgate & Hunter, 1962; Shleeva et al., 2010). Recent data, however, cast doubt on the assumption of such 'inactive' latent state, as there is constant metabolic activity within the TB bacilli (Zumla et al., 2011). Evidence about the role of molecular chaperones and intercellular signalling molecules in control of metabolic activity and composition of the cell wall has been provided by Henderson et al. (2010).

From the view point of the L-cycle theory, a transition of mycobacteria from acid-fast to non-acid fast state, along with appearance of polymorphic cell wall deficient cells, occurs in response to stress. L-forms develop through several stages and result in formation of polymorphic or coccoid fast growing cells. The initial phase of L-conversion probably corresponds to an "invisible" stage, where bacteria cease forming colonies on solid media and growing in liquid media. We suppose that formation and persistence of giant L-forms structures (filaments, syncytia and "mother" cell) sheltering and embodying many individuals inside a common envelope, represents a unique mechanism of survival and may resemble "invisible" or cryptic state of L-form development. However, at some point of L-form development, these giant spherical or filamentous forms start to disintegrate and are no longer visible, giving place to an abundance of granular and coccoid forms, which sometimes become the prevailing elements within L-population. Coccoid forms of mycobacteria, called "mycococcus", were obtained *in vitro* by Csillag in 1964. Mycococcii were grown from *M. tuberculosis* and were similar to the morphology of staphylococci (Csillag, 1964). Genetic analysis of mycobacterial coccoids however, performed by us through amplification of 16SrRNA gene fragment, 16S-23S rRN gene Internal Transcribed

Spacer sequences and IS *6110* PCR, verified them as *M. tuberculosis* (n. d.). DNA sequencing analysis is currently in progress (n. d.).We consider that the invisible L-conversion phase is followed by a state of active reproduction of non- acid fast and non-recognizable as mycobacteria L-forms usually with coccoid morphology. Taken together, these data may argue that the curious morphology and growth characteristics of mycobacterial L-forms, their extremely different habit of existence define them as specific type of unrecognizable and hidden persisters. As seen in Fig.6, L-form conversion cycle of mycobacteria is schematically outlined with emphasis on ability of different L-structures to form colonies. In this sense, L-form persistence phenomenon substantially differs from the current understanding for latency as persistence of few ''non-replicating''or ''dormant'' bacteria.

3.3 Growth characteristics and colonial morphology

In contrast to classical tubercle bacilli, we found that L-form variants, obtained after nutrient starvation stress of *M. tuberculosis in vitro,* grew and developed colonies phenomenally faster, mimicking rapidly replicating bacteria.

The morphology of growths underwent progressive changes, which resulted in formation of typical L-form colonies with "fried egg" appearance (Fig.7 b, c).

As pointed out by other authors, dark centers of "fried egg" colonies usually consist of dense granular elements, which are deeply embedded in the medium but at the periphery of the colony large pleomorphic bodies are frequently found (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981). The shape of L-form colonies resulted from the variety of individual structural units and the way that they divided (Mattman, 2001). It should be pointed out that fully developed L-type colonies appeared between 36 and 48 hours after plating on Middlebrook semisolid agar, in contrast to control *M. tuberculosis* microcolonies.

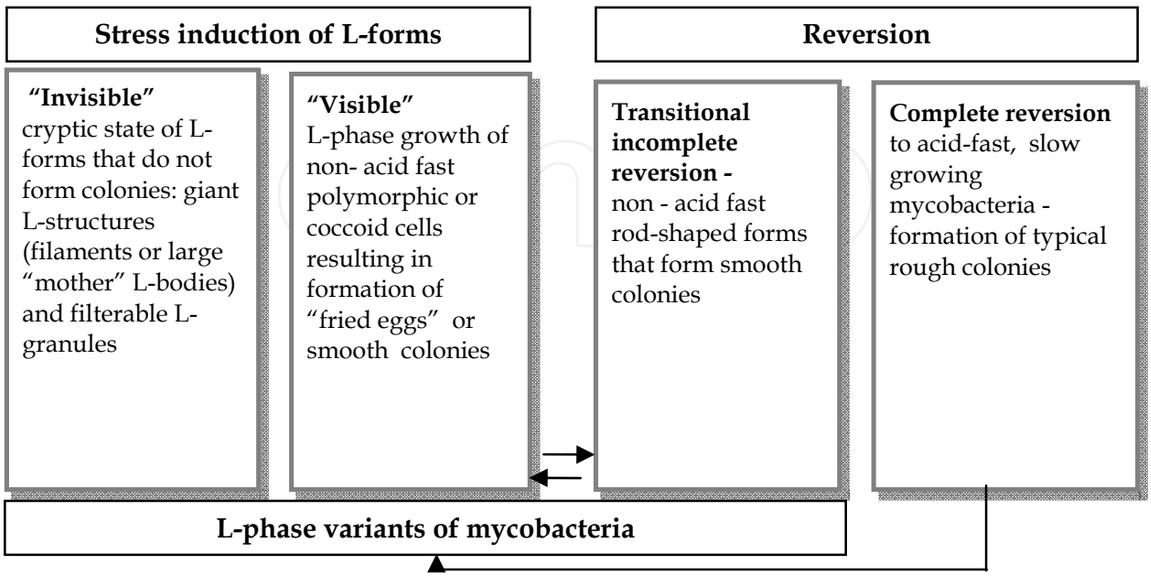
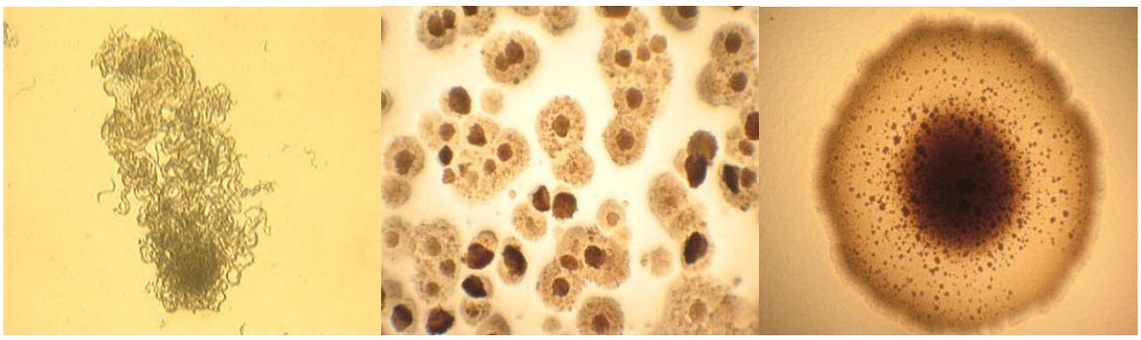


Fig. 6. Morphological phases during L-form conversion and reversion of mycobacteria.

We suggest that the lack of cell walls and easier permeation of nutrients is the reason for the unique ability of mycobacterial L- forms to grow faster in comparison to classical tubercle bacilli. Pla Y Armengol (1931) found that a large inoculum of tubercle bacilli grows rapidly on all routine media, appearing as large L-body spheres and also vegetated mycelia. In our study, L-form variants were adapted without difficulties to grow on conventional nutrient agar. Light and electron microscopy also provided interesting results about the appearance of non-acid fast coccoid cell morphology of stressed *M. tuberculosis*, that support observations of other authors. The appearance of non-acid fast coccoids in cultures of mycobacteria has been reported by others in the beginning of the last century but the phenomenon was not clearly explained and proven at that time (Csillag, 1964; Juhasz, 1962; Xalabander, 1958;). More surprising was the fact that mycobacterial coccoid L-forms not only mimicked the morphology of staphylococci or other coccus- shaped bacteria, but also exhibited extremely rapid growth and colonial development in contrast to classical TB bacilli (n. d.). Coccoid cells were initially mistaken by us as contaminants, but the specific DNA testing (amplification of 16SrRNA gene fragment, 16S-23S rRNA gene Internal Transcribed Spacer sequences, IS*6110* PCR and DNA sequencing analysis) identified them as *M. tuberculosis* (n. d.). We suppose that non-acid fast coccoid L-form variants of mycobacteria resulted probably from the more regular mode of multiplication, synchronization and stabilization of L-form cells under specific condition of cultivation. Thus, it can be presumed why such coccoid forms of *M. tuberculosis* remain often unrecognized or are mistaken for contaminants.



a. b. c.

Fig. 7. Light microscopy of (a) control *M. tuberculosis* rough microcolony and (b, c) typical "fried eggs" shaped colonies of *M.tuberculosis* L-forms obtained after nutrient starvation stress (n. d.).

Standard plating techniques are often inadequate for accurate enumeration of microbial dormant forms, because some of them may be in a "nonculturable" state (Shleeva et al., 2010). When it comes to L-forms**,** they are considered to be both "difficult-to-cultivate" and "difficult-to-identify". Because of their altered morphology and fully changed bacterial life cycle, L-forms are difficult to be identified in clinical materials. The isolation of arising *in vivo* L-forms is generally possible only with special procedures ensuring their enrichment and resuscitation to actively growing state i.e. having an ability to form colonies (Michailova et al., 2000a; Zhang et al., 2001; Zhang, 2004). The use of specially supplemented liquid and semisolid media, as well special techniques, like so called "blind" passages, are absolutely necessary for isolation of L-forms from specimens (Michailova et al., 2005; Markova et al., 2008a).

3.4 Yin-Yang hypothesis for co-existence of classical and L- forms within natural mycobacterial population

The Yin-Yang hypothesis is based on the idea that classical and cell wall deficient forms co-exist within natural mycobacterial populations. The Chinese concept of the complementary alternating forces of Yin and Yang provides opportunities to better understand the natural phenomenon of heterogeneity and correspondence between both subpopulations in mycobacteria. The Yin-Yang point of view, suggesting the hypothesis for coexistence of classical and cell wall deficient forms, is illustrated in Fig.6.

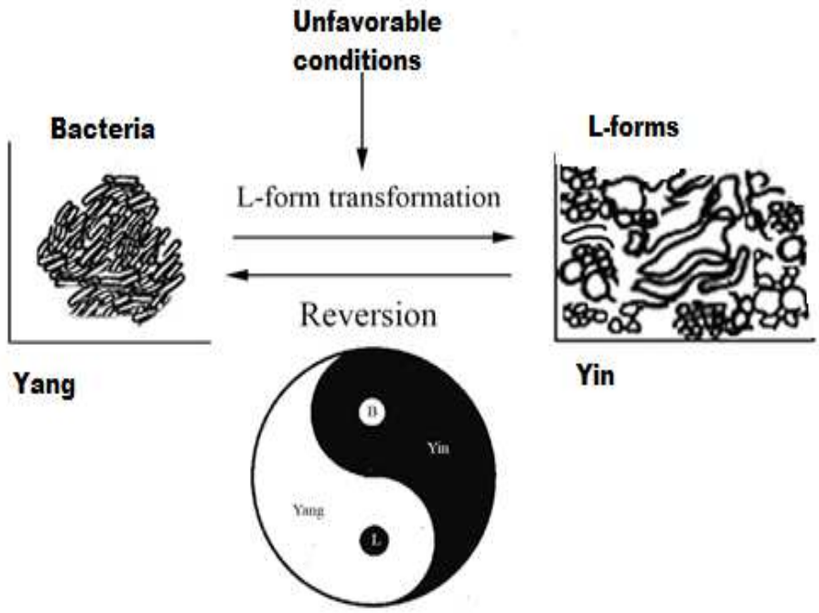


Fig. 8. Alternating "Yin-Yang"–life phases of classical walled bacteria and cell wall deficient L-forms: "Yang" - monomorphic population of classical rod shaped bacteria under optimal conditions ensuring yield and nutrients for growth and cell division; "Yin"- polymorphic population of L-forms. Polymorphism ensures survival advantages and arsenal of various notoriously resistant to environmental assaults L-form structures under unfavorable conditions.

The morphological diversity within bacterial populations is often related to heterogeneous environments observed under natural conditions. Population-based morphological variability and cell wall deficiency of *M. tuberculosis* might be considered as a natural phenomenon ensuring the adaptive strategy of this pathogen for environmental change (Markova, 2009; Mattman, 2001). By making use of our Yin-Yang concept, by utilizing available scientific data on this subject and our own findings, we try to figure out how classical walled and cell wall deficient subpopulations interact under different conditions. We found that both classical walled and cell wall deficient L-forms coexist within clinical strains of *M. tuberculosis* freshly isolated from sputum of patients has been demonstrated in our study (Michailova et al., 2005). This finding supports the concept that natural mycobacterial population usually consists of prevalent classical forms and small numbers of L- forms. There is data reporting about coexistence of classical walled and cell wall deficient

L-forms within natural populations of other bacteria as well as about relations of concurrence and interference between them under different conditions (Boris *et al.*, 1969; Fodor and Roger, 1966). Extreme morphological plasticity of bacteria has been found to provide survival advantages (Justice *et al.*, 2008; Young, 2007). It is assumed that L-forms occur along with resistance to factors that trigger their appearance (Prozorovski, 1981).

We found that under stress *in vitro* and *in vivo* (Markova et al, 2008a) the balance within mycobacteria was shifted in favor of cell wall deficient forms and the population continued to exist, replicating predominantly as L-forms. Our *in vitro* experiments aimed to induce L-conversion of *M. tuberculosis,* by means of nutrient starvation stress (n. d.). Once the process in favor of L-form development was induced and shifted, further selective separation of L-form variants was made, based on the unique ability of mycobacterial L- forms to grow faster in comparison to classical tubercle bacilli due to the lack of cell walls and easier permeation of nutrients. Selection of mycobacterial L-forms was achieved technically through transfers of stressed mycobacterial cultures at weekly intervals on semisolid Middlebrook agar. Due to their growth advantage, mycobacterial L-form variants became the prevailing subpopulation, overgrowing classical TB bacilli during the performed five passages, which resulted in isolation of L-form cultures. As has been demonstrated in our previous study, similar L-form transformation of *E. coli* was found to appear under conditions of starvation and, more surprisingly, after lethal heat stress (Markova et al., 2010). However, it has been found that cell wall deficient forms of *E. coli* developed slower than classical walled forms. In contrast with interactions between both subpopulations during L-form transformation of *E. coli*, we recognized the opposite relations between classical and L-forms in mycobacteria, which were strongly influenced by the special biochemical structure and physiology of TB bacilli.

4. Formation and persistence of mycobacterial L-forms in vivo

Animal models for the study of tuberculosis include guinea pigs, mice, rabbits and nonhuman primates. Despite the difficulty in modeling human latency in experimental animals, the understanding of both host and microbial factors that contribute to the establishment and maintenance of a persistent *M. tuberculosis* infection has progressed and the information gathered is pertinent to human latent tuberculosis (Flynn & Chan, 2001). Formation of *M. tuberculosis* L-forms *in vivo* were demonstrated by means of biological experiments on guinea pigs (Li, 1990; Markova et al., 2008b ; Ratnam &Chandrasekhar, 1976; Snitinskaia et al., 1990;), mice (Belianin et al., 1997) and rats (Markova et al., 2008a).

In our study, we established a rat model of experimental tuberculosis that produces mycobacterial cell-wall deficient forms *in vivo* (Markova et al, 2008a). Although rats are not a common animal model for TB research, we attempted, on basis of our previous experience with other bacterial L- form experimental infections (Markova et al, 1997; Michailova et al., 2000), to use the capability of these animals to exhibit high innate resistance to infections, thus ensuring inhibition of classical bacterial forms and inducing the occurrence of cell-wall deficient forms. After intraperitoneal and intranasal infection with *M. tuberculosis*, samples from lung, spleen, liver, kidney, mesenterial and inguinal lymph nodes and broncho-alveolar and peritoneal lavage liquid were taken and plated simultaneously on Löwenstein-Jensen medium or inoculated into specially supplemented for L-forms Dubos broth at weekly intervals over five weeks. Mycobacterial L-form cultures were isolated throughout

the whole period of the experiment, including the last two weeks, when typical mycobacterial colonies consisting of classical bacilli were not isolated on Löwenstein-Jensen medium. If we had used only the classical isolation procedure with Löwenstein-Jensen media alone, we would have been led to falsely believe that mycobacteria were completely eliminated. However, mycobacteria continued to persist as L-forms at the late stage of infection. We believe that the established by us rat model of experimental tuberculosis can mimick latent infection.

Mycobacteria can convert to cell wall deficient forms (L-forms) inside macrophages. After intraperitoneal administration of BCG, samples of peritoneal lavage fluid from guinea pigs were obtained at day 1, 14 and 45. In order to study whether and how *M. bovis* BCG can transform in L-forms and persist *in vivo*, series of events during interaction of live BCG bacilli with peritoneal macrophages in guinea pigs were evaluated and observed by transmission electron microscopy (Markova et al, 2008b). At the late intervals of infection, an interesting phenomenon of L-form formation inside macrophages was observed. The percent of the formed L-forms at day 14 was about 15% and at day 45, we did not find any BCG bacilli with normal morphology - all observed bacteria were in L-form state. Examination of BCG bacilli inside macrophages revealed morphological peculiarities typical of cell wall deficient bacterial L-forms, as well as different modes of L-form multiplication. As shown in Fig. 9 (d, e, f), pleomorphic and relatively large BCG L-form bodies were found inside vacuoles which were found to persist for a long time inside macrophages due to the ineffectual phagocytosis, digestion and clearance**.** Fusion of small phagosomes containing L-forms and formation of larger ones was seen as well . Additional point of interest was the observation that many mitochondria (M) with enlarged size and endoplasmic reticulum dilation (ER) were clustered closely and around L-forms . The observed process of organelle translocation appeared to be related to the intracellular life of L- forms - survival and multiplication. Microbial digestion, respectively a process of complete phagocytosis of L-forms, was not observed. Some intra-phagosomally located L-forms inside macrophages were surrounded by multi-membranes (Fig. 9 f) and so packed within membranes they were released to the extracellular space. The observed cycle of L-form attachment and engulfment by new phagocytes at the late stage of infection suggests that L-forms probably exploit apoptotic-like pathway as means of returning to the extracellular environment and for subsequent rounds of new entry and uptake by macrophages. Obviously, such apoptosis-like pathway may protect L-forms from humoral and cellular host defense factors during their trafficking from intracellular to extracellular compartment and vice versa. It is generally assumed that apoptosis has developed as a host defence mechanisms against infection, but it is not completely clear what advantages apoptosis can provide to bacteria (Keane et al., 2000; Riendeau & Kornfeld, 2003; Rosenberger & Finlay, 2003). A number of authors have presented evidence that cell-wall defective variants can be formed within macrophages (Mattman, 2001; Michailova et al., 2000a; Thacore & Willett, 1966). Thacore and Willett (1966) have reported about formation of spheroplasts of *M. tuberculosis* within tissue culture cells.

Since *M. bovis* BCG is an attenuated live strain, little is known about how long it can survive in the vaccinated individuals . Reports about detection and isolation of BCG bacilli from patients with AIDS many years after vaccination (Armbruster et al., 1990; Reynes et al., 1989; Smith et al., 1992) give rise to questions about the mechanisms by which BCG bacilli persist

in vivo for a long time. As far as cell wall deficiency facilitates the bacterial survival under unfavorable conditions, L-forms of different bacterial species have been shown to survive and persist for an extended period inside macrophages due to the ineffectual phagocytosis, digestion and clearance (Markova et al., 1997; Michailova et al., 1993; Michailova et al., 2000b; Michailova et al., 2007). The finding that of all the bacteria, L-forms predominate and are crucial to the survival of mycobacteria *in vivo* (Mattman, 2001; Michailova et al., 2005) needs to be taken into account when developing and putting in use new viable mycobacterial vaccines, especially considering that L-forms of *M. bovis* BCG bacilli have been found in the blood of persons vaccinated against TB with BCG vaccine (Xalabarder, 1958). This provides us with insight of the importance of L-form conversion phenomenon for the behavior, persistence and safety of live BCG vaccines.

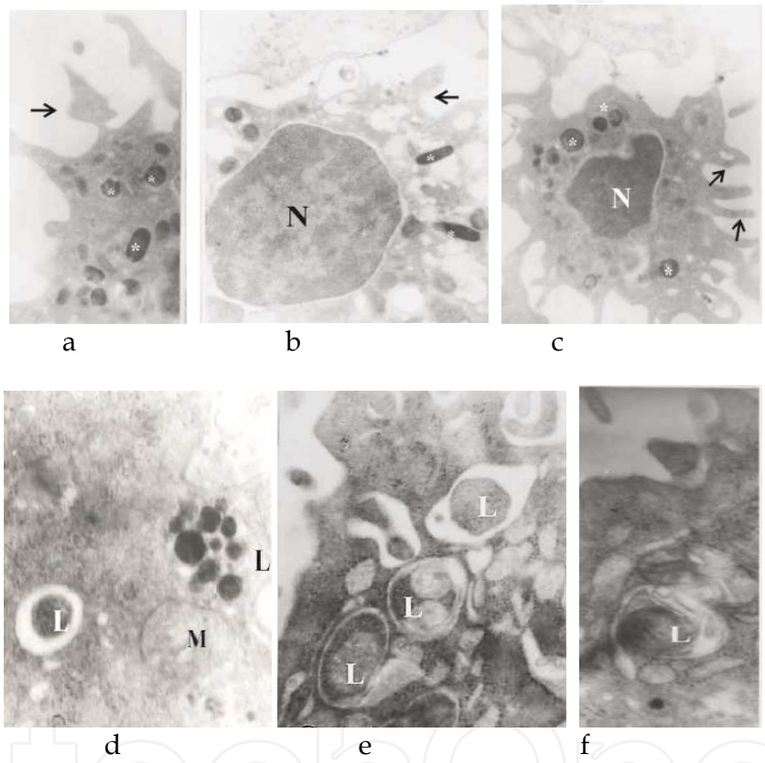


Fig. 9. Formation of BCG L-form cells (L) within the vacuoles in guinea pigs peritoneal macrophages**: a, b , c** -at day 1 after BCG installation, the interactions of BCG bacilli (*) with peritoneal cells demonstrated initial phases of phagocytosis including attraction, adhesion and attachment of bacteria to the phagocytes and processes of bacterial enclosing and engulfment; **d, e, f**- at day 45 after BCG installation, formation of BCG L-form cells (L) within the vacuoles near to mitochondria (M); **d**, **e** - L-form multiplication inside macrophages; **f**- BCG L-form large bodies , surrounded by multi membranes; Bar = 0.5 µm (Markova et al., 2008)

5. Reversion of mycobacterial L-forms to classical TB bacilli

Reversion of L-forms to normal parental bacteria is an important property, which is inducible by changing the condition of cultivation *in vitro* or occurs spontaneously *in vivo*

under favorable for the pathogen circumstances. Mattman defined the essential factors for reversion, the most popular of which are omission of the inducing agent, changes in nutrition, concentrating populations, inoculation in to experimental animals and others (Mattman, 2001). Of special interest is the reversion stimulated by products from microbes. Rathham & Chandrasekhar reported about reversion of filterable variants of tubercle bacillus from sputum by culturing with Freund's adjuvant (Rathham & Chandrasekhar, 1976). Although atypical forms are genetically programmed to develop a cell wall, it is not yet clear how compromised cell wall deficient bacteria mobilize the energy necessary for reversion to bacterial walled phase. It is interesting to note that the reversion of mycobacterial L-forms to normal TB bacilli appeared to be more difficult and slower, when compared to other bacteria.

There is a widespread assumption, which perceives the dormant state of *M. tuberculosis* as a *reversible* state or as ability of mycobacteria to reverse into active state and to reactivate the disease (Shleeva et al., 2010). Recently, it has been found that bacteria possess a specific system for autoregulation of growth and development, which participates in control of cell differentiation at the level of regulation of the functional activity of subcellular components and of the cell as a whole (Shleeva et al., 2010). Resuscitation-promoting factors have also been identified and their role in latency and reactivation of tuberculosis have been investigated (Biketov et al., 2007; Zhang et al., 2001;). Five genes encoding Rpf-like proteins have been found in *M. tuberculosis* genome, which may act in reactivation of "nonculturable " forms of *M. tuberculosis* (Kana et al., 2008; Mukamolova et al., 2002; Tufariello et al., 2004). Shleeva et al. (2003) found that cell-free culture liquid of an exponential-phase *Mycobacterium tuberculosis* culture or the bacterial growth factor Rpf exerted a resuscitating effect, substantially increasing the growth capacity of the nonculturable cells in liquid medium. During resuscitation of nonculturable cells, a transition from ovoid to rodlike cell shape occurred.

6. Clinical significance and role of mycobacterial L-forms

Arguments for and against significance of L-forms as infecting and persisting agents, respectively their role in human and animal diseases, are limited because of difficulties in their isolation, cultivation and identification. However, a lot of papers, reviews (Allan et al., 2009; Beran et al., 2006; Domingue and Woody, 1997; Domingue, 2010; Gumpert & Taubeneck, 1983; Onwuamaegbu et al., 2005; Zhang, 2004) and several monographs (Domingue, 1982; Mattman, 2001; Prozorovski et al., 1981), support the concept that L-forms can be induced *in vivo*, can persist there for a significant span of time and can be the cause for latent, chronic and relapsing/recurrent infections, as well as for diseases of unknown infectious-allergic or autoimmune origin.

However, of all the bacteria, L-forms predominate and are crucial to the survival of *M. tuberculosis in vivo.* Therefore they are thought of as carriers of a tubercular constitution (Mattman, 2001). The understanding of cell wall deficiency in *M. tuberculosis* may occur as consequence of a long-lasting interaction with the host and as a strategy to ensure its survival and persistence *in vivo* is still limited, as mycobacteria are quite difficult to detect, especially when in their viral-like, cryptic state. Although the mechanisms of spontaneously occurring *in vivo* cell wall deficient forms are difficult to explain, many authors have considered that mycobacteria, undergoing L-form transformation, are of clinical significance

for the incidence of relapses and are a prognostic unfavorable indicator (Berezovski & Salobai, 1988; Dorozhkova. et al., 1989, Dorozhkova et al., 1990; Khomenko et al., 1980). Observation of atypical, non-acid fast and cell wall deficient forms of *M. tuberculosis* in patient specimens suggests their occurrence *in vivo*. Kochemasova succeeded in isolating *M. tuberculosis* L-forms from cerebrospinal fluid, from resected sections of different organs of tuberculosis patients, as well as from urine of patients with renal tuberculosis during long lasting chemotherapy (Berezovski & Golanov, 1981; Kochemasova et al., 1970; Kochemasova, 1975). L-variants of *M. tuberculosis* were observed during antibacterial therapy of tuberculosis meningitis by Kudriavtsev et al. (1974). Of special interests were the reports by different authors about isolation of *Mycobacterium tuberculosis* L-forms from sputum and caverns of patients with pulmonary tuberculosis (Takahashi, 1979a, 1979b; Tsybulkina, 1979;). Zhu et al. (2000) found cell wall deficient forms of *M. tuberculosis in* biological material,particularly sputum and blood from patients with pulmonary tuberculosis. The first report of L-forms from *Mycobacterium scrofulaceum* infection, occurring in an 11 –year- old boy, was made by Korsak (1975). L-colonies consisting of non- acid fast coccoids and large spheres grew from autopsy materials (dermal lesions, brain, spleen, kidney, lung and intesties), sometimes making syncitya and reverting to acid fast bacilli.

Regardless of the huge progress in TB research and the development of new molecular technologies, pathogenesis of latent tuberculosis is still not well understood. The dynamic hypothesis of Cardona (2009) suggests that latent tuberculosis infection is caused by the constant endogenous reinfection of latent bacilli. Considering this hypothesis, constant "escape" of bacilli from granulomas before fibrosis is the primary source of bacteria, reactivation would never occur after a specific time period, unless the host suffered an immunosuppressive episode (Cardona & Ruiz-Manzano, 2004). Of special interest is the finding that foamy macrophages are able to maintain a stressful environment that keeps the bacilli in non-replicating state, but on the other hand, allow them to escape from granulomas, making them more resistant to future stressful conditions (Cardona et al., 2000; Cardona et al.; 2003; Cardona, 2009).

Currently, asymptomatic latent tuberculosis is defined not by identification of bacteria, but by host immune response tests. Although individuals with latent tuberculosis harbor viable bacteria, it is difficult to identify them (Young et al., 2009; Manabe & Bishai, 2000). Among the unresolved mysteries of latent tuberculosis is the nature and anatomical situation of persisting tubercle bacilli (Grange 1992). The common observation that acid-fast bacilli are frequently absent in smears is an indication that pathology may result from *in vivo* propagation of cell wall deficient mycobacteria (Domingue, 1982; Judge& Mattman, 1982). Thus, if diagnosis by finding these forms (cell wall free, non acid-fast persisting bacilli) becomes practice, it may have valuable application in diagnosis of latent tuberculosis.

There are many tuberculous syndromes in which the aetiology is occult or imitative of other diseases (Domingue, 1982; Judge & Mattman, 1982). Traditional concept of the mycobacterial aetiology of sarcoidosis and especially the assumption that cell wall deficient forms rather than bacillary are involved has been supported by several reports. Varying acid fast spindle-shaped or yeast-like structures, termed *pleomorphic chromogens*, and cell wall deficient forms of *M. tuberculosis* complex were detected in lymph node tissue from subjects with sarcoidosis (Alavi and Moscovic, 1996; Moscovic,1978). Cantwell also suggested that acid-fast organisms, found in skin lymph nodes and lung tissue from patients with

sarcoidosis, were mycobacterial cell wall deficient forms (Cantwell, 1982a,b). Judge & Mattman grew mycobacterial cell wall deficient forms (predominantly coccoid forms, larger L forms and short acid-fast rods) from blood of patients with sarcoidosis (Judge & Mattman, 1982). Polymerase chain reaction (PCR) was used to detect mycobacterial DNA in clinical samples from patients with sarcoidosis and in half the sarcoidosis patients was found *M tuberculosis* DNA (Saboor et al, 1992). A report, describing the molecular characterization of *M. tuberculosis* complex isolates from patients with sarcoidosis and tuberculosis, showed that half of the isolates from sarcoidosis patients did not resemble the spoligotypes of the isolates from patients with tuberculosis (Gazouli et al., 2005). Cell wall-defective mycobacteria were isolated also from skin lesions and cerebrospinal fluid of patients with sarcoidosis and identified to be *M. a. paratuberculosis* or other *M. avium-intracellulare* complex members (El-Zaatari et al., 1996). A relationship between cell wall deficient forms of *M. a. paratuberculosis* and Crohn's disease has been found by some authors, although this aetiological agent has not yet been conclusively proven (Hermon-Taylor and Bull, 2002; Hulten et al., 2001 a, b, c; Sechi et al. ,2001; Schwartz et al., 2000).

Future research in the field of cell wall deficiency in mycobacteria promises an increased accent on its association with latent and persistent bacterial state, which should be supported with modern molecular biological evidences. In order to better understand the nature of L-conversion phenomenon, it will be important to correlate *in vitro* with *in vivo* (experimental animals and patients) findings.

Since many researchers do not believe in existence of L-conversion phenomenon in mycobacteria, molecular genetic studies are relatively scarce (Hulten et al., 2000a,b; Lu et al, 2009; Melnikoava &, Mokrousova, 2006; Vishnevskaia et al. , 2001; Wang et al., 2007; Wall et al., 1993). On the other hand, L-forms are "difficult-to-identify" by most of the standard DNA-based tests, probably due to their unusual life style and irregular division. The relative scarcity and rather inaccessibility of the genetic material in L-forms make them generally difficult for genetic studies. De Wit & Mitchison (1993) indicated that mycococci derived from mycobacteria did not exist. The authors examined stored cultures of the mycococcus form of *M. bovis* BCG and *M. phlei* which were prepared by Csillag in 1972 and 1969 and found that restriction fragment patterns of the DNA of the variant forms and the parent mycobacteria were not similar. Traag et al (2009) also found no evidence that mycobacteria produced free-living "spores" (i.e cocci). However, the verification of L-forms isolated from experimental animals as genuine *M. tuberculosis* but not as contaminating bacteria became possible in our study, with species - specific spoligotyping test (spacer oligonucleotide typing technique) and after some modification of the initial steps in preparing the L-form cultures (Markova et al., 2008a). Spoligotyping results provided interesting insight into the occurrence of certain polymorphisms, *i.e.* insertion or deletion of spacer signals in some of the L-form isolates. In our laboratory, we have also gained much experience in experiments to obtain stable mycobacterial L-forms *in vitro* and have already developed a reproducible protocol, which allows obtaining sufficient biomass of L-cultures to get enough DNA. Under screening is a spectrum of the most examined genes for detection, identification and characterization of *Mycobacterium tuberculosis* complex in stable mycobacterial L-form cultures. The next necessary step after gene screening would be the sequencing analysis, in order to understand what kind of genetic events happen during L-transformation and which mechanisms lead to cell wall deficiency.

7. Conclusion

In conclusion, tubercle bacilli may use L-form conversion as unique adaptive strategy to survive and reproduce under unfavorable conditions in hosts. Possibility for persistence and reversion of L-forms to classical TB bacilli *in vivo* elaborates on some specific aspects of L-conversion phenomenon and link them to the mechanisms at play in latent tuberculosis. Morphologically modified and non-acid fast L-forms of mycobacteria are difficult to identify and often remain unrecognized, or are mistaken for contaminants. "L-form persistence phenomenon" of actively growing and propagating by unusual modes cell wall deficient cells differs definitively from the current understanding for latency as persistence of a few''non-replicating'' or ''dormant'' bacteria. Mycobacterial L-forms give rise to many unsolved questions concerning their biology and behavior *in vivo*, as well as about the genetic regulatory mechanisms leading to their appearance. Cell wall deficiency in mycobacteria remain an interesting topic that needs to be re-examined in the context of modern molecular biology.

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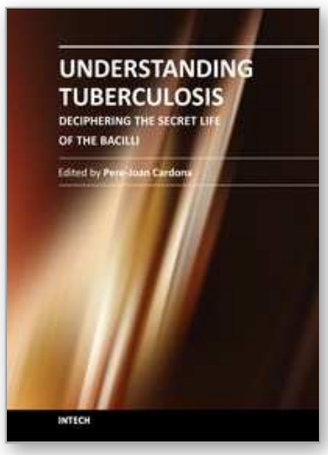

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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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